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REMARKS

Claims 1-9 are pending in the instant application. Claims 7-9 have been withdrawn from consideration by the Examiner and subsequently canceled without prejudice by Applicants in this amendment. Claims 1-6 have been rejected. Reconsideration is respectfully requested in light of the following remarks.

I. Finality of Restriction Requirement

The Examiner has made final the Restriction Requirement mailed March 26, 2003, agreeing however, to include Groups I and II in the instant application. Thus, in an earnest effort to advance the prosecution of this case, Applicants have canceled claims 7-9 without prejudice. In light of the finality of this Restriction Requirement, Applicants reserve the right to file a divisional application to the canceled subject matter.

II. Objection to Title

The Examiner has objected to the title suggesting that it is not descriptive of the invention to which the claims are directed. Thus, in an earnest effort to advance the prosecution of this case, Applicants have amended the title to be descriptive

of the subject matter of the pending claims. Withdrawal of this objection is therefore respectfully requested.

III. Rejection of Claims 1-6 under 35 U.S.C. § 103(a)

Claims 1-6 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over Anderson (U.S. Patent 5,994,104) in view of Ruoslahti et al. (U.S. Patent 6,180,084). The Examiner suggests that it would have been obvious to one of ordinary skill in the art, at the time the invention was made to operably link interleukin-12 disclosed in Anderson et al. to a RGD-containing peptide protein described by Ruoslahti et al. to produce a fusion protein conjugate capable of binding to αv-integrin receptors on the surface of tumor blood vessels sufficient to destroy the blood vessels and cause tumor necrosis because Anderson et al. teaches that a rIL-12 has potent anti-tumor effects and Ruoslahti et al. teach that chemotherapeutic agents linked to a tumor homing molecule are more efficacious than administration of the agent alone, in treating a tumor.

Applicants respectfully traverse this rejection.

MPEP § 2143 states that to establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must

be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art to modify the reference or combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art references when combined must teach or suggest all the claim limitations. The cited combination does not meet these criteria.

As acknowledged by the Examiner, Anderson et al. does not teach interleukin-12 operably linked to a RGD-containing peptide that can be targeted to tumor cells. In fact, the fusion protein to which Anderson et al. refers is the fusion of two IL-12 subunits, not fusion of IL-12 to a different protein or peptide. Further, while there are teachings in Anderson et al. of coexpressing their IL-12 fusion protein with B7, there is no teaching or suggestion of creating a fusion protein of the IL-12 subunits and B7. Accordingly, this reference provides no motivation or suggestion to fuse an RGD-containing peptide to interleukin-12.

Ruoslahti et al. fails to remedy the deficiencies in the teachings of Anderson et al. since this reference also fails to provide any specific teaching or suggestion of fusion of IL-12 to a RGD-containing peptide.

Thus, there is no suggestion or motivation in the references themselves to combine their teachings.

Further, prior art teachings such as by Lieschke et al. (Nature Biotechnology 1997 15:35-40), a copy of which is provided herewith, show that activity of IL-12 is decreased in fusion proteins. Accordingly, the knowledge generally available to one of ordinary skill in the art teaches away from modifying these references or combining their teachings to produce a fusion protein of IL-12 and a RGD-containing peptide.

Further, neither the cited references nor what is known in the prior art (see e.g. Lieschke et al.) provide any reasonable expectation of success that a fusion protein of IL-12 operably linked to a RGD-containing peptide will maintain IL-12 anti-tumor activity or anti-angiogenic activity.

In addition, it has recently been demonstrated in references such as Reynolds et al. (Nature Medicine 2002 8:27-34), a copy of which is provided herewith, that αvβ3 and αvβ5 integrins are negative regulators of angiogenesis and that drugs targeting these receptors may act as agonists of angiogenesis as opposed to antagonists. Thus, if αvβ3 and αvβ5 block angiogenesis, binding of RGD would be expected to remove the negative regulation of αvβ3 and αvβ5, thereby increasing vessel growth. Accordingly,

based upon information available to one skilled in the art, one would reasonably expect the IL-12vp fusion protein of the present invention to exhibit decreased anti-tumor activity as compared to IL-12 and either stimulate angiogenesis through the RGD moiety or to see little change when compared to IL-12 treated mice or cells since the RGD angiogenic activity would be expected to negate the IL-12 anti-angiogenic activity, at least to some extent. Thus, the demonstrated ability of the fusion protein of the present invention to inhibit angiogenesis and tumor growth is completely unexpected.

Thus, since the cited combination of prior art references and what is generally known in the prior art provide no suggestion or motivation to combine the reference teachings and the cited prior art teachings provide no reasonable expectation of success with respect to the instant claimed invention, the cited combination of prior art cannot render obvious the instant claimed invention.

Withdrawal of this rejection of claims 1-6 under 35 U.S.C. § 103(a) is therefore respectfully requested.

IV. Conclusion

Applicants believe that the foregoing comprises a full and complete response to the Office Action of record. Accordingly, favorable reconsideration and subsequent allowance of the pending claims is earnestly solicited.

Respectfully submitted,

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Bioactive murine and human interleukin-12 fusion proteins which retain antitumor activity in vivo

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Interleukin-12 (IL-12) is unique amongst cytokines in being a disulfide-linked heterodimer of two separately encoded subunits (p35 and p40). We expressed single chain IL-12 proteins from retroviral constructs in which the two IL-12 subunits were linked by a 6–15 amino acid polypeptide linker, with deletion of the 22 amino acid leader sequence of the trailing subunit. The murine fusion protein IL-12.p40.L.Δp35 containing a (Gly,Ser)$_3$ linker was stably expressed, bioactive in vitro, and had an apparent specific activity comparable to that of native and recombinant IL-12. Western blotting confirmed that murine IL-12.p40.L.Δp35 retained the linking polypeptide sequences. The analogous human IL-12.p40.L.Δp35 fusion protein containing a Gly$_4$Ser linker was bioactive with an apparent specific activity comparable to recombinant human IL-12. In a preexisting CMS-5 tumor model, CMS-5 cells secreting either native or fusion protein forms of IL-12 prolonged survival and led to complete tumor regression.

Keywords: interleukin, cytokine, fusion protein, retrovirus, tumor immunotherapy

Interleukin-12 (IL-12) augments innate and cellular immunity in many ways potentially advantageous for the treatment of infectious and malignant disease[1]. Preclinical studies have evaluated the effects of systemic administration of recombinant IL-12 heterodimer[2–4] and initial clinical trials are in progress[5]. Alternative approaches have relied on genetic strategies for IL-12 production, using retroviral, adenoviral, and direct DNA gene transfer technologies. For example, fibroblasts and tumor cells transduced to secrete IL-12 have been shown to be highly effective therapies for preexisting tumors in several murine models[6–8], and clinical studies of these genetically-based approaches have commenced[9]. However, the heterodimeric structure of IL-12 substantially complicates construction of vectors for IL-12 production, because the expression of bioactive IL-12 requires expression of two separate genes and correct heterodimer assembly. This has previously been achieved in various ways: (1) cotransfection of two plasmids separately expressing each subunit gene[4], (2) a single plasmid with IL-12 p35 and p40 expression cassettes in tandem array[4], (3) by retroviruses containing internal ribosome entry site (IRES) sequences for the expression of both subunits from single polycistronic transcripts[10–12], or (4) by a recombinant adenoviral vector with the subunit cDNAs separately inserted into early region 1 and E3 respectively[13].

We now report the expression of a bioactive single chain murine IL-12 fusion protein (IL-12.p40.L.Δp35) with high specific in vitro bioactivity and which retained the potent antitumor activity of IL-12 in vivo. IL-12.p40.L.Δp35 comprised the p40 subunit linked by a (Gly,Ser)$_3$ linker to the p35 subunit from which the first 22 amino acids were deleted. The analogous human IL-12 fusion protein also retained high in vitro bioactivity.

Results

Expression of bioactive murine IL-12 fusion proteins. A series of constructs were built in the SFG retroviral vector[14] for the expression of native murine IL-12 and IL-12 fusion proteins (Fig. 1A). SFG retroviral vectors containing either the IL-12 p35 or p40 cDNA (pSFG.IL-12.p35 and pSFG.IL-12.p40) were built for cotransfection or coinfection studies. For the expression of native IL-12 from a single retroviral vector, dicistronic SFG vectors were built with an internal ribosome entry site (IRES) sequence between the IL-12 p35 and p40 cDNAs (pSFG.IL-12.p35.IRES.p40 and pSFG.IL-12.p40.IRES.p35). For the expression of IL-12 fusion proteins; SFG vectors were built with the IL-12 p35 and p40 cDNAs linked in both orders, and with the presumed 22 amino acid secretory leader sequence deleted from the trailing cDNA (pSFG.IL-12.p35.L.Δp40 and pSFG.IL-12.p40.L.Δp35). The cDNAs were linked with a 45 base pair linker encoding the 15 amino acid (Gly,Ser)$_3$ linker of Huston et al.[15] The sequences encoding the linkers as sucessfully cloned are shown in Figure 1B.

Constructs were compared for their ability to direct bioactive IL-12 expression in a side-by-side screening experiment: CMS-5 tumor cells were infected with retroviruses generated by transfection of BOSC producer cells and medium conditioned by infected cells assayed for bioactive and immunoreactive IL-12 (Table 1). To standardize for possible differences in transfection and infection efficiency within this screening experiment, the dimensionless deduced parameter *relative specific activity* (ratio of ng/ml determined by bioassay and immunoassay) was calculated. This parameter standardizes the bioactivity to the amount of expressed protein, assuming equivalent immunoreactivity of all forms of IL-12 in the IL-12 p75 ELISA.

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Table 1. Transient expression of murine IL-12 retroviral constructs[a].

Construct	Proviral copy number in infected cells[b]	IL-12 in conditioned medium (ng/ml)[c] Bioassay	IL-12 p75 ELISA	IL-12 p40 ELISA	Relative specific activity[d]
Nil	ND	<0.1	ND	<0.1	—
pSFG	ND	<0.1	ND	<0.1	—
pMFG.lacZ	ND	<0.1	ND	<0.1	—
pSFG.IL-12.p35	0.1	<0.1	ND	<0.1	—
pSFG.IL-12.p40	0.3	<0.1	<0.25	192	—
pSFG.IL12.p35 and pSFG.IL-12.p40 (cotransfection)	0.1/0.1[e]	21.6	15.6	52.5	1.78
pSFG.IL12.p35 and pSFG.IL-12.p40 (sequential infection)	0.3/0.3[e]	362	203	205	1.38
pSFG.IL-12.p35.IRES.p40	<0.1[f]	5.3	4.3	2.5	1.23
pSFG.IL-12.p40.IRES.p35	<0.1[f]	2.7	2.3	11.5	1.17
pSFG.IL-12.p35.L.Δp40	0.4[f]	0.9	88.9	23.6	0.01
pSFG.IL-12.p40.L.Δp35	0.2[f]	436	204	93.9	2.14

ND=not determined. Contruct designations as defined in Figure 1. [a]Constructs were screened side-by-side in one experiment. For full methodological details see Experimental protocol. [c]24 h conditioned media were collected starting on day 3 after seeding 5×10^5 CMS-5 cells/6 cm tissue culture dish/5 ml. For full methodological details see Experimental protocol. [d]Relative to a plasmid copy number control of 13.5 pg of pSFG.IL-12.p35.L.p40, equimolar to 1 copy/genome loaded per lane. [d]Ratio of bioactive:immunoreactive IL-12 concentrations in ng/ml (determined by capture bioassay and IL-12p75 ELISA), i.e., a dimensionless value independent of transfection or infection efficiency. Comparisons between these values assume equivalent immunoreactivity for the different forms of IL-12. [e]Copy number given separately for p35/p40 retroviruses. [f]Mean of results from Southern blots probed with IL-12.p35 and then IL-12.p40 cDNA probes.

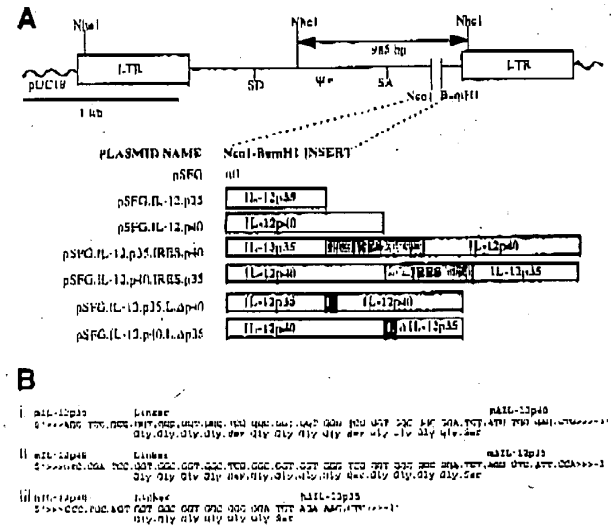

Figure 1. Constructs for the expression of native murine IL-12 and IL-12 fusion proteins. (A) Schematic diagram of retroviral vectors. SD, splice donor; SA, splice acceptor; LTR, long terminal repeat; IRES, internal ribosome entry site; L, linker. (B) Sequences of linkers in murine (m) and human (h) IL-12 fusion protein cDNAs. (i) mIL-12.p35.L.Δp40; (ii) mIL-12.p40.L.Δp35; (iii) hIL-12.p40.L.Δp35.

Uninfected CMS-5 cells and cells infected with control vectors or vectors directing the expression of single IL-12 subunits did not result in production of bioactive IL-12. Bioactive native IL-12 production resulted from infection of target cells with mixed retrovirus populations resulting from the cotransfection of producer BOSC cells with pSFG.IL-12.p35 and pSFG.IL-12.p40 plasmids, or from the sequential infection of target cells with these retroviruses, and from infection of cells with both IRES-containing retroviruses. The relative specific activity of the native heterodimeric IL-12 resulting from these retroviruses was in the range 1.17–1.78. Comparison of the results of the ELISAs specific for either the p75 IL-12 heterodimer or the p40 subunit (both free and heterodimeric) suggested that the pSFG.IL-12.p40.IRES.p35 vector directed the expression of excess p40 subunit (Table 1). Since excess p40 subunits have potential to form homodimers that antagonize IL-12[16-18], the pSFG.IL-12.p35.IRES.p40 vector was selected for subsequent studies.

The constructs directing expression of linked IL-12 subunit cDNAs resulted in bioactive IL-12 fusion protein production (Table 1). Although significant levels of immunoreactive fusion protein were detected resulting from pSFG.IL-12.p35.L.pΔ40, it was of low relative specific activity (0.01). The pSFG.IL-12.p40.L.Δp35 vector appeared superior in every regard: An average number of proviral integrants (0.2 copies/genome) resulted in expression of high levels of a protein of high relative specific activity (2.14). In a separate determination, the bioactivity of the fusion protein produced by retrovirally transduced 3T3 cells was 2.5×10^6 U/mg, similar to that of Chinese hamster ovary (CHO) cell-expressed recombinant heterodimeric mIL-12[19] (specific activity 2.3–2.5×10^6 U/mg; data not shown), although this comparison assumes equivalent immunoreactivity of the two IL-12 forms in the ELISA used for protein quantitation. Thus the vector directing expression of the IL-12.p40.L.Δp35 fusion protein was selected for subsequent studies.

Western blot analysis confirmed that pSFG.IL-12.p40.L.Δp35 directed the expression of an IL-12 fusion protein that did not rely on disulfide bonds for its integrity (Fig. 2). Recombinant mIL-12 and native IL-12 synthesized from pSFG.IL-12.p35.IRES.p40 ran as a 70–80 kDa doublet under non-reducing conditions, and some free p40 subunit was present in both these preparations. The IL-12.p40.L.Δp35 fusion protein was expressed as a doublet of slightly higher molecular weight than the native form (Fig. 2A). Whereas recombinant and native IL-12 dissociated under reducing conditions, the IL-12.p40.L.Δp35 polypeptide remained intact (Fig. 2B), demonstrating that this protein did not rely on disulfide bonds for the integrity of its polypeptide sequence, and that the linking peptide sequences had not been excised during posttranslational processing of the fusion protein.

Expression of a bioactive human IL-12 fusion protein. An analogous human form of the IL-12.p40.L.Δp35 fusion protein was also found to be bioactive with high specific activity. The full sequence of Huston et al.[19] encoding the (Gly,Ser)$_5$ linker was found to be unstable in precursor constructs with numerous varied deletions identified in the linker sequences of recombinant plasmids, but a stable deletion mutant encoding an in-frame (Gly$_4$Ser linker was recovered (linker sequence given in Fig. 1B)

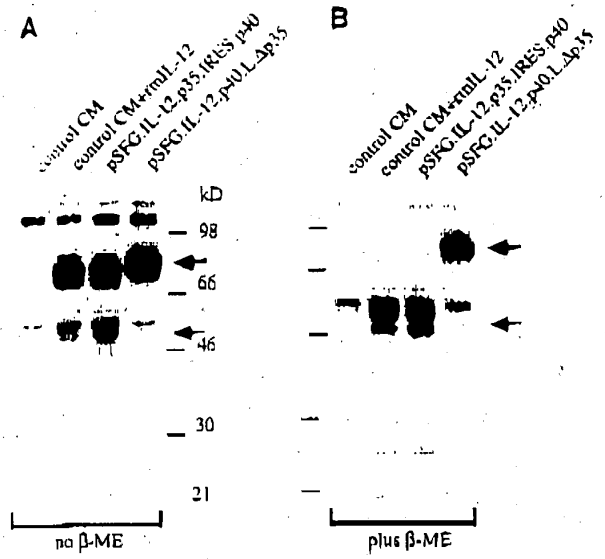

Figure 2. Western blot analysis of recombinant heterodimeric and fusion protein forms of murine IL-12 under non-reducing (A) and reducing (B) conditions. CM, conditioned medium; rmIL-12, recombinant murine IL-12; β-ME, β-mercaptoethanol. Dark arrow, p75 IL-12; gray arrow, p40 subunit. Contruct designations are defined in Figure 1, and indicate the retroviruses used to infect cells for the preparation of native and fusion protein IL-12-containing conditioned media.

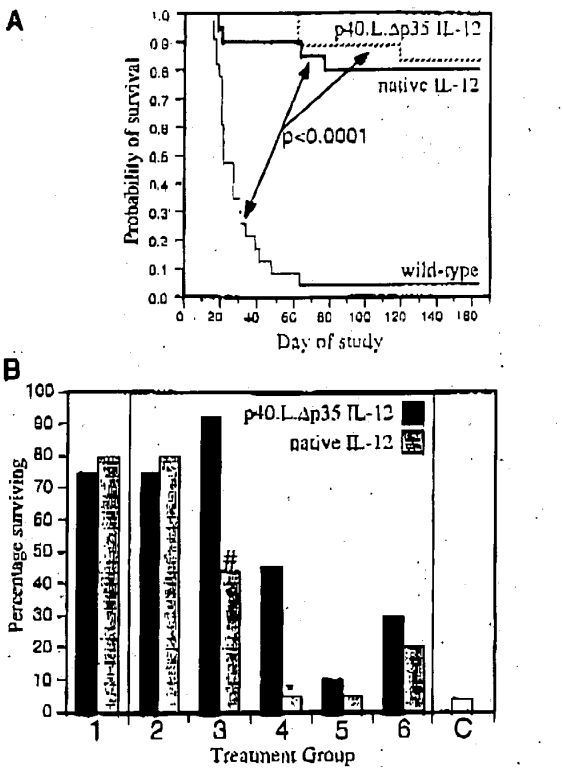

Figure 3. Relative in vivo antitumor activity for established day 14 CMS-5 tumors treated with CMS-5 cells secreting either native or fusion protein forms of IL-12. (A) Kaplan-Meier survival plots for mice treated with 5×10⁶ wild type (thin line, n=24), native IL-12-secreting (thick line, n=20) or single-chain IL-12-secreting (broken line, n=20) CMS-5 cells. (B) Titration of the relative in vivo antitumor activity of CMS-5 cells transduced to secrete either native or fusion protein forms of IL-12. Mice bearing 14-day tumors initiated with 4×10⁴ CMS-5 cells were treated with either 10⁷ IL-12-secreting cells (Group 1), or 5×10⁶ cells of which 100, 20, 4, 0.8, 0.16 or 0% of cells were from the stock population of native or fusion protein IL-12-secreting CMS-5 cells (Groups 2–6 and C respectively). 20 mice per group except group 3 (n=25) and control group C (n=24); #, p=0.0003; *, p=0.004. Data are pooled from two separate experiments and survival was scored at day 162.

(designated pSFG.hIL-12.p40.L.Δp35). 3T3 cells were infected with SFG.hIL-12.p40.L.Δp35 retrovirus expressed transiently from transfected BOSC producer cells and conditioned medium collected and assayed for bioactive and immunoreactive human IL-12. Bioactive hIL-12 was produced with a specific activity of 1.7×10^8 U/mg (mean of two determinations in parallel), compared to $1.2–2.0 \times 10^6$ U/mg for CHO cell-expressed recombinant hIL-12[28] (data not shown), although again, this comparison assumes equivalent immunoreactivity of the two IL-12 forms in the ELISA used for protein quantitation.

In vivo antitumor activity of murine IL-12 fusion protein. IL-12-expressing tumor cells have been reported to be effective therapy for preexisting tumors in several transplantable murine tumor models[2]. Hence we evaluated the relative in vivo anticancer activity of tumor cells transduced to express either native IL-12 (pSFG.IL-12.p35.IRES.p40) or the IL-12 fusion protein (with pSFG.IL-12.p40.L.Δp35).

Stable retrovirus producer clones were generated by transfection of pSFG.IL-12.p35.IRES.p40 and pSFG.IL-12.p40.L.Δp35 constructs into ψ-CRE and ψ-CRIP producer cells. Stable producer clones selected for their efficient transduction of 3T3 cells for IL-12 production were used to infect tumor cell populations in vitro. IL-12 secretion by tumor cell populations was measured by bioassay of conditioned media from irradiated transduced cells (Fig. 2). Infection of CMS-5 fibrosarcoma cells with ψ-CRE-derived ecotropic retrovirus resulted in secretion of high levels of native and fusion protein IL-12. ψ-CRIP-derived amphotropic retrovirus was necessary to achieve comparable levels of IL-12 secretion by B16 melanoma cells. (Assay of conditioned media from 10^6 non-irradiated cells documented equivalent or higher levels of IL-12 production.) Transfected unselected tumor cell populations maintained equivalent levels of native or fusion protein IL-12 production during 6 weeks of propogation in vitro, indicating that here was no significant negative selection against IL-12 secreting cells in vitro.

CMS-5 cells secreting either form of IL-12 were effective therapy for established CMS-5 tumors. Tumors were established with 4×10^4 CMS-5 cells, resulting in a palpable tumor incidence of 90% on day 14 (median mean tumor diameter 4 mm; range 0–12.5 mm). Overall long-term survival of mice with CMS-5 tumors was ≥80% when cells transduced to secrete either form of IL-12 were administered in four weekly doses commencing on day 14, compared to 4% for control mice treated with wild-type tumor cells (Fig. 3A). Eighty-one percent of palpable tumors in mice treated with cells secreting IL-12 regressed completely, compared to 0% of tumors in control mice.

It was possible that there may have been a difference in the antitumor efficacy of IL-12-secreting cell populations that would be evident at submaximal levels of IL-12 secretion. Populations of tumor cells secreting different amounts of IL-12 were generated by mixing wild-type CMS-5 cells with diminishing proportions of IL-12-secreting cells and evaluated for therapeutic efficacy in mice with preexisting CMS-5 tumors (Fig. 3B). CMS-5 cells secreting p40.L.Δp35 IL-12 retained maximal in vivo antitumor

Table 2. Stable transduction of tumor cells for IL-12 production.

Tumor cell line	Retroviral producer cell line cell line/clone number	Provirus copy number	Bioactive IL-12 production μg/10⁶ irradiated cells/ 48 hr (no. of assays)
CMS-5	nil	0	<0.0005[a]
	CRE.IL-12.p35.IRES.p40/4	1.4	4.24 ± 0.73 (34)[c]
	CRE.IL-12.p40.L.Δp35/43	0.5	7.49 ± 1.42 (26)[c]
B16	nil	0	<0.0005[a]
	CRE.IL-12.p35.IRES.p40/2	1.1	0.057 ± 0.002 (3)[b]
	CRIP.IL-12.p35.IRES.p40/2	4.7	1.10 ± 0.26 (9)[c]
	CRIP.IL-12.p40.L.Δp35/22	1.8	1.86 ± 0.55 (10)[d]
	CRIP.IL-12.p40.L.Δp35/32	ND	11.01 ± 2.67 (2)[c]

ND=not determined. Results from conditioned media assayed undiluted[a], or diluted 1:100[b], 1:1,000[c] or 1:10,000[d].

activity when diluted 1:5 in wild-type cells (i.e., the treatment dose of 5×10^6 CMS-5 cells comprised 1×10^6 cells from the IL-12.p40.L.Δp35-secreting population and 4×10^6 wild-type CMS cells), whereas the cells secreting native IL-12 had only 50% of maximal in vivo antitumor activity at this dose level. However, since the p40.L.Δp35 IL-12-secreting CMS-5 cells secreted 3–5 fold more IL-12 bioactivity than the native IL-12-secreting CMS-5 cells (based on in vitro bioassay of media conditioned by the cells used for the four treatment doses in these experiments), this difference was not evident when the data were standardized for the amount of secreted IL-12 bioactivity. No therapeutic difference was evident between cells secreting the two forms of IL-12 whether 1, 2, 3, or 4 weekly therapeutic doses of 5×10^6 IL-12-secreting cells were administered.

Discussion

We have described the expression of novel bioactive murine and human IL-12 fusion proteins. The murine fusion protein IL-12.p40.L.Δp35 has a specific activity comparable to that of native and recombinant IL-12, and retains antitumor activity in vivo. The analogous human IL-12 fusion protein is also bioactive. Although the single chain IL-12 fusion proteins we describe resulted from an attempt to simplify the construction of retroviral vectors for IL-12 production, they potentially have wide additional application. The cDNAs encoding the human or mouse IL-12 fusion proteins circumvent the difficulties[9–13] of expressing two separate proteins to produce IL-12 and hence would be convenient for use in plasmid, adenoviral, and other expression systems under evaluation for cancer and infectious disease gene therapy. A particular theoretical advantage of single chain IL-12 expression is the elimination of the possibility of excess free p40 subunit synthesis, a potential problem since excess free p40 subunits have the capacity to form homodimers that function as specific IL-12 antagonists in both murine and human in vitro systems[14–18]. Furthermore, compared to native IL-12, the IL-12 fusion protein itself may have a different spectrum of relative potencies over the range of pleiotropic IL-12 activities, possibly resulting from a different affinity for IL-12 receptors, or from different pharmacolobiology and pharamokinetics in vivo, although we have not yet evaluated these possibilities. For the commercial manufacture of heterodimers such as IL-12, the large scale production of a single chain form may be simpler than that of the heterodimer, particularly because there is no need to include steps to purify the active moiety from contaminating subunit forms.

No initial presumption was made about the optimal order of components in the polypeptide backbone or the length of the linker, since there was no 3-dimensional structural information about IL-12 available at the outset. The (Gly,Ser)$_4$ linker of Huston et al.[19] was arbitrarily chosen for its flexibility[20], and also because it had been used previously in constructing single chain antibodies[21] and PIXY-321 (ref. 22), a linked form of human granulocyte macrophage colony stimulating factor and interleukin-3 already in clinical use. Cloning practicalities and a propensity for intra-linker deletion to occur during cloning influenced the final cDNA constructed for hIL-12.p40.L.Δp35 (an intra-linker deletion also occured in the construction of the PIXY-321 cDNA from the same linker sequence[22]). The basis of the different relative specific activities of the murine IL-12 fusion proteins expressed is likely to be structural, although the fusion protein did not alter the combination of sequences recently shown to be critical for the binding of murine p35 to the murine IL-12 receptor[23]. It is possible that the deletion of the leader sequence of the trailing cDNA might contribute to the relative potency and stability of the IL-12.p40.L.Δp35 protein relative to some of the other numerous IL-12 fusion protein forms possible, by minimizing the bulk of nonessential sequences in this region of the otherwise correctly folded fusion protein, and by removing potential protease sites.

A potential disadvantage of incorporating an extraneous linker sequence in a therapeutic compound is that it may present immunogenic epitopes in vivo. The immunogenicity of the (Gly,Ser)$_4$ linker was not a consideration in its design for single chain antibodies and in vivo studies have been too limited for this to be known. The deletion variant linker Gly,SerGly,Ser in PIXY-321 (ref. 22) is also of unknown immunogenicity, although there is one preliminary report of antibody development and reduced efficacy of this compound with repeated administration[24]. However, the specificity of these antibodies has not been reported in detail and may have been against epitopes on either IL-3, GM-CSF, or the linker, and the presence of the powerful adjuvant GM-CSF in this fusion moiety may have unusually enhanced its inherent immunogenicity such as was seen with anti-idiotype-GM-CSF fusion proteins[25]. An assessment of the immunogenicity of the IL-12 fusion proteins based on in vivo administration of purified recombinant material will be of interest.

The unselected populations of IL-12-producing tumor cells we made by infection with SFG-based retroviruses secrete 10–70 fold more IL-12 than the best achieved by others using IRES-containing retroviral constructs and selection for infected tumor cells[5,6,8]. The recently isolated producer CRIP.IL-12.p40.L.Δp35/32 resulted in 900-fold greater IL-12 production by B16 cells than had previously been achieved for this tumor cell line[7]. Enrichment for infected cells by selection was not possible with our constructs since they did not contain a positive selectable marker, but our tumor cell populations averaged 0.5–4.7 proviral integrants per cell indicating high retroviral infectivity. Since we achieved high production of both native and single-chain IL-12, the difference is not explained by characteristics of the single chain IL-12 moiety per se although there was a trend for cells transduced to express the single chain IL-12 to secrete higher levels of IL-12 bioactivity despite lower rates of proviral integration.

With the high level of IL-12 secretion attained, we were able to demonstrate that the antitumor effect of IL-12-secreting CMS-5 cells exhibited a dose-response relationship in the preexisting CMS-5 tumor model, and that for survival and tumor regression endpoints the plateau of a dose-response relationship had been achieved. Notably, CMS-5 cells secreting a 25-fold reduction of IL-12 (extrapolated to be approximately 300 ng/10^6 irradiated cells/48 hr) had no in vivo antitumor activity. These observations are consistent with previous reports[6,7] indicating that high levels of IL-12 secretion may be required in some tumor models for antitumor effects to be seen, and suggest that where subtle or minimal effects have been observed in models using tumor cells secreting low amounts of IL-12, this may have resulted from a low level of IL-12 production.

The availability of murine and human single chain IL-12 fusion

proteins of high specific in vitro bioactivity and potent antitumor activity in vivo provides an opportunity to expand the development of IL-12-based therapeutic strategies. This is particularly so for genetically-based strategies, since the cDNAs encoding these single-chain forms of IL-12 substantially simplifies the construction of vectors for IL-12 production in plasmid, retroviral, adenoviral and other vector systems.

Experimental protocol

Cell lines. ψ-CRIP[26], ψ-CRE[27] and BOSC[28] producer cells, 3T3 cells, and B16[29] and CMS-5[29] tumor cell lines were propagated as previously described[30].

Retroviral vector construction. Murine (m) and human (h) IL-12 p35 and p40 cDNA clones in pBluescript (Stratagene, La Jolla, CA) (pBS.IL-12.p35 and pBS.IL-12.p40) were a gift of Ueli Gubler (Hoffmann-La Roche, Nutley, NJ). The cDNAs corresponded to the published sequences[31–33], except that the sequences of the murine p35 and murine and human p40 cDNAs surrounding the translational initiation ATG were changed to 5′-ACCATGG-3′, in all three cases altering the second codon from TGT (cysteine) to GGT (glycine) and creating an NcoI restriction enzyme site. The internal ribosome entry site (IRES) sequence used was derived from the encephalomyocarditis virus[34] as previously used[35]. Linker sequences were synthesized as sense and antisense oligonucleotides spanning the sequence of Huston et al.[11] encoding the (Gly,Ser)$_4$ linker, and adjacent sequences between convenient overhanging restriction enzyme sites in the 3′ and 5′ ends of the IL-12 subunit cDNAs, deleting the stop codon of the leading IL-12 subunit cDNA. Linker oligonucleotides were annealed, phosphorylated and ligated into dephosphorylated plasmids. In some cases, intra-linker deletions occurred during cloning (see Fig. 1B). The SFG retroviral vector used was derived from MFG[36,37a] by the introduction of two point mutations which create an NheI site and cause the premature termination of the gag open reading frame sequences present in MFG (sequence details provided elsewhere[11]). SFG was propogated in the pUC19 plasmid and the cDNA inserts for expression were cloned between its unique NcoI and BamH1 sites (the BamH1 site was filled where necessary) using conventional techniques[37] (Fig. 1). Inserts in final versions of constructs were as follows (nucleotide numberings are from the published reports[31–33]): pSFG.IL-12.p35: p35 cDNA as a NcoI-EcoR1[filled] fragment of pBS.IL-12.p35; pSFG.IL-12.p40: p40 cDNA as a NcoI-BamH1 fragment of pBS.IL-12.p40; pSFG.IL-12.p35.IRES.p40: p35 cDNA as a NcoI-EcoR1 fragment of pBS.IL-12.p35, IRES cDNA as a EcoR1-NcoI fragment, p40 cDNA as a NcoI-BamH1 fragment of pBS.IL-12.p40; pSFG.IL-12.p40.IRES.p35: p40 cDNA as a NcoI-BamH1 fragment of pBS.IL-12.p40, IRES cDNA as a BamH1-NcoI fragment, p35 as a NcoI-EcoR1[filled] fragment of pBS.IL-12.p35; pSFG.IL-12.p35.L.Δp40: p35 cDNA from the NcoI site of pBS.IL-12.p35 to nt 771 (i.e., deleting the stop codon), linker as shown in Figure 1B(i), p40 cDNA from nt 101 to the EcoR1[filled] site of pBS.IL-12.p40 (i.e., deleting the first 22 codons of p40); pSFG.IL-12.p40.L.Δ.p35: p40 cDNA from the NcoI site of pBS.IL-12.p40 to nt 1039 (i.e., deleting the stop codon), linker as shown in Figure 1B(ii), p35 cDNA from nt 193 to the EcoR1[filled] site of pBS.IL-12.p35 (i.e., deleting the first 22 codons of p35); SFG construct for the expression of human IL-12.p40.L.Δ.p35: hIL-12 p40 cDNA from the NcoI site of pBS.hIL-12.p40 to nt 997 (i.e., deleting the stop codon), linker as shown in Figure 1B(iii), hIL-12 p35 cDNA from nt 270 to the PpuMI[filled] site of pBS.hIL-12.p35 (i.e., deleting the first 22 codons of p35 following the second translational initiation codon). Constructs were sequenced across all cloning junctions to determine the nucleotide sequence of the linker and adjacent subunit regions.

Expression of IL-12 proteins and collection of conditioned media. For the side-by-side screening of a panel of constructs (Table 1), BOSC producer cells were plated at 2×10^6 cells per 6 cm tissue culture dish and transfected [CaPO$_4$] transfection with the various constructs as previously described[30]. Twenty-four hours after transfection, the medium was replaced with 5 ml fresh medium. Virus-containing supernatants were collected 24 h later, filtered (0.45 μm, Gelman Sciences, Ann Arbor, MI) and polybrene added (final concentration 8 μg/ml). CMS-5 cells were infected with 2.5 ml of virus-containing supernatant for 4 h (CMS-5 cells had been plated at 5×10^5 cells per 6 cm tissue culture dish the previous day). The remaining 2.5 ml of supernatant was frozen at −70°C and used for a second 4 h infection of the CMS-5 cells the following day. To collect IL-12-containing conditioned medium, the medium was replaced the following day with 5 ml fresh medium which was harvested 24 h later, filtered (0.2 μm) and frozen at −70°C for later assay. The infected CMS-5 cells were then lysed, and genomic DNA prepared for later analysis. For selected constructs, stably transfected ψ-CRE and ψ-CRIP producer clones[30] were generated as previously described[30], screening individual producer clones for those with the greatest capacity to transduce 3T3 cells to produce bioactive IL-12. Retrovirus-containing supernatants from selected stable producer clones were used to infect tumor cell lines to generate populations of IL-12-secreting tumor cells. Tumors were transduced by four sequential 4 h infections in the presence of polybrene (8 μg/ml). To facilitate infection of B16 cells with ecotropic ψ-CRE-derived retrovirus, pre-treatment with 2-deoxy-D-glucose was used according to a method developed by D. Lindemann (University of Würzburg, Germany, unpublished) based on observations of Kai et al.[38] For comparative determinations of IL-12 production by tumor cells (Table 2), 10^6 irradiated cells were plated in 10 ml of culture medium and conditioned media harvested after 18 h, filtered (0.2 μm), and stored at −70°C until assayed.

IL-12 bioassays and ELISAs. Levels of IL-12 bioactivity in conditioned media (both serum-free and containing 10% fetal calf serum) were determined using the murine IL-12 splenocyte proliferative bioassay as described[11]. Samples were assayed at 1:1–1:10,000 dilutions and bioactivity determined in comparison to the linear part of a standard curve using recombinant murine (rm) IL-12 (gift of M. Gately, Hoffmann-La Roche) constructed over the range 20–5000 pg/ml rmIL-12 in the test sample. Curve fitting was performed using Deltagraph 4.0 (Deltapoint, Monterey, CA). All conditioned media samples contained demonstrable inhibitory activity; values reported were taken from the most dilute sample falling within the linear range of the standard curve, generally a 1:100 or 1:1,000 dilution. To calculate the relative specific activity (defined as the ratio of ng/ml determined by bioassay and immunoassay), a capture IL-12 bioassay was used as this was not complicated by the effects of inhibitory substances in conditioned media. Antibody capture bioassays and ELISA for murine and human IL-12 were performed as previously described[30,39]. The murine IL-12 p40 ELISA, which detects both heterodimeric and free p40, used the p40-specific 5D9 antibody to coat plates, whereas the heterodimer-specific mouse IL-12 p75 ELISA used the heterodimer-specific 9A5 antibody for coating. The mouse p40-specific antibody 5C3 was used in both ELISAs for detection of captured mouse IL-12 p40 or IL-12 p75. Anti-mouse IL-12 monoclonal antibodies were provided by D. Presky (Hoffmann-La Roche). A commercially available human IL-12 p75 ELISA (R&D Systems, Minneapolis, MN) was used for initial screening for human IL-12.p40.L.Δp35 production. Specific activity calculations for hIL-12.p40.L.Δp35 were based on bioactivity determinations in the antibody capture bioassay[39] and protein quantitation using the ELISA that employs the heterodimer-specific antihuman IL-12 antibody 20C2 as a capture antibody for human IL-12 p75[39].

Proviral copy number determination. Proviral copy number was determined by Southern blot analysis[37]. Genomic DNA (10 μg) digested by NheI was separated by electrophoresis in 0.8% agarose gels, transferred to a nylon membrane and separately probed with full-length ^{32}P-labelled IL-12 p35 or p40 cDNA fragments from pBS.IL-12.p35 and pBS.IL-12.p40, with validated stripping between hybridizations with each probe. Control lanes were based on 1 proviral copy in 10 μg genomic DNA equalling 13.5 pg of pSFG.IL-12.p35.L.p40, and probe hybridization to the two endogenous genes served as a further internal control.

Western blot analysis. Serum-free conditioned media from wild-type CMS-5 cells or CMS-5 cells expressing native IL-12 or the IL-12 fusion protein were collected, filtered (0.2 μm), and stored at −70°C. The conditioned media were concentrated 20–30 fold (Centricon 10, Amicon, Beverly, MA), and samples containing 20 μg total protein run on separate 10% polyacrylamide gels with or without 10% β-mercaptoethanol. The primary antibody was a polyclonal goat anti-rmIL-12 antibody (gift of D. Presky, Hoffmann-La Roche), the secondary antibody a peroxidase-conjugated donkey anti-goat F(ab′)$_2$ fragment at a 1:50,000 dilution (Jackson Immunoresearch Labs, West Grove, PA), and the "Renaissance" detection system (DuPont NEN, Wilmington, DE) was used. A preliminary analysis indicated a fourfold-greater signal from conditioned media containing single chain IL-12 and therefore for the Western blot shown in Figure 2, 5 μg total protein from this conditioned medium was loaded. The control lanes were conditioned media from wild-type cells without or spiked with 50 ng recombinant murine IL-12.

In vivo tumor model. The protocol was approved by the Massachusetts Institute of Technology Animal Care Committee. To initiate CMS-5 tumors, syngeneic 6–8 week old female BALB/c mice from Jackson Laboratories (Bar Harbor, ME) were injected with 4×10^5 viable CMS-5 cells in 0.5 ml Hank's balanced salt solution (HBSS, Gibco, Grand Island, NY) subcutaneously on their back. On day 14, tumor size and incidence was documented

and a dose of 5×10^5 irradiated (135 Gy, ^{137}Cs source discharging 1.24 Gy/min) treatment cells administered subcutaneously in 0.5 ml HBSS in the left inguinal region. Treatment cells were wild-type CMS-5 cells, or unselected populations of CMS-5 cells transduced to express native IL-12 or the IL-12.p40.L.Δp35 fusion protein, or, for the experiment shown in Figure 3B, various mixtures of wild-type and IL-12 secreting cells. Treatment cells were administered to surviving mice weekly for 4 subcutaneous doses in alternating inguinal regions. Tumor size was monitored as the mean of two perpendicular tumor diameters, and mice were killed when the sum of two perpendicular tumor diameters exceeded 30 mm, or rarely, if tumors ulcerated (≥90% of non-survivors were sacrificed because of tumor bulk).

Statistics. Results are given as mean ± standard error. JMP 3.1.5 software (SAS Institute, Cary, NC) was used for statistical analyses and construction of Kaplan Meier survival curves. Rare deaths due to incidental causes (e.g., failure to recover from anaesthetic) were treated as censored events for the construction of survival curves, but scored as non-survival in categorical classifications. Survival differences were evaluated with the Wilcoxon rank sum test, and the chi-squared test was used for categorical variables.

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Enhanced pathological angiogenesis in mice lacking β_3 integrin or β_3 and β_5 integrins

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Inhibition of $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin function has been reported to suppress neovascularization and tumor growth, suggesting that these integrins are critical modulators of angiogenesis. Here we report that mice lacking β_3 integrins or both β_3 and β_5 integrins not only support tumorigenesis, but have enhanced tumor growth as well. Moreover, the tumors in these integrin-deficient mice display enhanced angiogenesis, strongly suggesting that neither β_3 nor β_5 integrins are essential for neovascularization. We also observed that angiogenic responses to hypoxia and vascular endothelial growth factor (VEGF) are augmented significantly in the absence of β_3 integrins. We found no evidence that the expression or functions of other integrins were altered as a consequence of the β_3 deficiency, but we did observe elevated levels of VEGF receptor-2 (also called Flk-1) in β_3-null endothelial cells. These data indicate that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are not essential for vascular development or pathological angiogenesis and highlight the need for further evaluation of the mechanisms of action of α_v-integrin antagonists in anti-angiogenic therapeutics.

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, involves coordinated endothelial-cell proliferation, migration and tube formation. This process is influenced both by growth factors, such as vascular endothelial growth factor (VEGF), and by cell adhesion molecules such as integrins[1,2]. Angiogenesis is a hallmark of cancer, as well as various ischemic diseases such as retinopathy of prematurity[3], implying that anti-angiogenic drugs are likely to be of importance in the treatment of these diseases. Elucidating the precise molecular mechanisms of angiogenic regulation is therefore important in determining rational strategies for such anti-angiogenic approaches.

VEGF has been identified as a major angiogenic factor acting through endothelial cell-specific receptors, including VEGF receptor-2 (VEGFR-2; also called Flk-1)[4]. The importance of the VEGF/VEGFR-2 system in angiogenesis is supported strongly by the lack of vascular development and early embryonic lethality in mice both heterozygous for and deficient in VEGFR-2 (refs. 4-6). In many tumors and ischemic diseases, VEGF production is elevated, inducing adult pathological angiogenesis and further emphasizing the importance of VEGF in neovascularization. Strategies to block VEGF and VEGF receptor signaling and function have resulted in significant inhibition of tumor angiogenesis and such reagents are presently in clinical trials[1,7,8]. Several members of the integrin family are also implicated in angiogenesis[9-18]. The largest body of data has linked $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (both receptors for vitronectin and other extracellular matrix molecules) with blood-vessel development[11-18]. Particular attention has been paid to the role of $\alpha_v\beta_3$ integrin in angiogenesis as it is prominent on proliferating vascular endothelial cells[13,14]. Furthermore, blockade of $\alpha_v\beta_3$ integrin with monoclonal antibodies or low-molecular-weight antagonists inhibits blood-vessel formation in a variety of *in vivo* models[17], including tumor angiogenesis[11-13] and neovascularization during oxygen-induced retinopathy[19]. In a recent report, a single small-molecule inhibitor of both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins inhibited tumor angiogenesis in animal models[20]. Taken together, these inhibition data suggest critical roles for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in angiogenesis, and highlight their importance as potential targets in anti-angiogenic therapy. In fact, the $\alpha_v\beta_3$-integrin antagonist, Vitaxin, is presently in clinical trials[21].

In contrast with these inhibitor studies, mice lacking α_v, β_3 or β_5 integrins exhibit extensive developmental angiogenesis[22-24]. All α_v-null mice have extensive sprouting angiogenesis and develop normally until embryonic day 9.5 and approximately 20% of them survive to birth[22]. β_3-null mice are both viable and fertile and developmental angiogenesis, including postnatal neovascularization of the retina, appears to be β_3-independent[23]. β_5-null mice are also viable and fertile and have no defects in wound healing, suggesting that adult angiogenesis is unaffected in these animals[24]. These results indicate that the precise role of α_v integrins in angiogenesis is likely to be more complex than initially thought and raise the question of the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in adult pathological angiogenic processes. Using genetically defi-

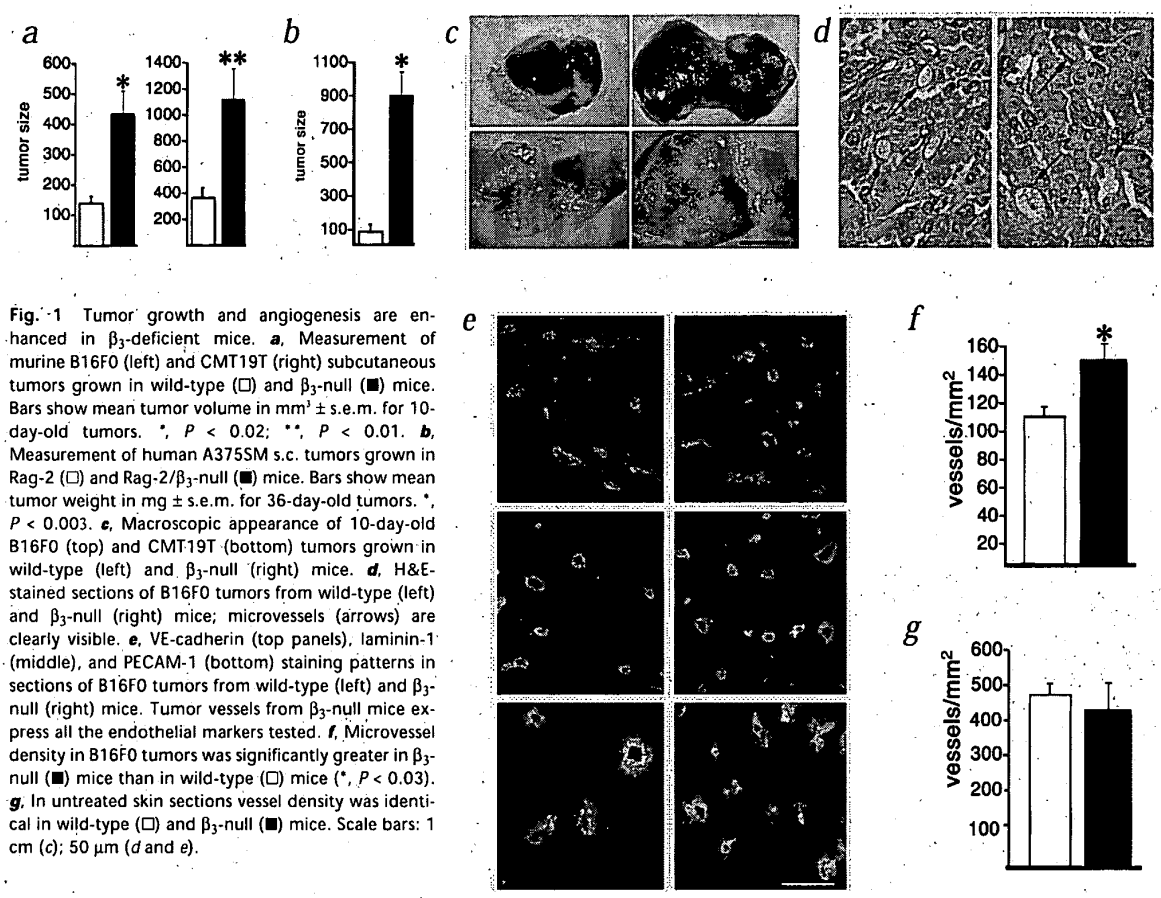


Fig. 1 Tumor growth and angiogenesis are enhanced in β_3-deficient mice. ***a***, Measurement of murine B16F0 (left) and CMT19T (right) subcutaneous tumors grown in wild-type (□) and β_3-null (■) mice. Bars show mean tumor volume in $mm^3 \pm$ s.e.m. for 10-day-old tumors. *, $P < 0.02$; **, $P < 0.01$. ***b***, Measurement of human A375SM s.c. tumors grown in Rag-2 (□) and Rag-2/β_3-null (■) mice. Bars show mean tumor weight in mg ± s.e.m. for 36-day-old tumors. *, $P < 0.003$. ***c***, Macroscopic appearance of 10-day-old B16F0 (top) and CMT19T (bottom) tumors grown in wild-type (left) and β_3-null (right) mice. ***d***, H&E-stained sections of B16F0 tumors from wild-type (left) and β_3-null (right) mice; microvessels (arrows) are clearly visible. ***e***, VE-cadherin (top panels), laminin-1 (middle), and PECAM-1 (bottom) staining patterns in sections of B16F0 tumors from wild-type (left) and β_3-null (right) mice. Tumor vessels from β_3-null mice express all the endothelial markers tested. ***f***, Microvessel density in B16F0 tumors was significantly greater in β_3-null (■) mice than in wild-type (□) mice (*, $P < 0.03$). ***g***, In untreated skin sections vessel density was identical in wild-type (□) and β_3-null (■) mice. Scale bars: 1 cm (*c*); 50 μm (*d* and *e*).

cient mice, we have tested the requirements for β_3, or the combination of β_3 and β_5 integrins, in adult neovascularization by monitoring pathological angiogenesis. Here we report the following results: 1) tumor growth and angiogenesis not only are supported, but were even enhanced in β_3-deficient and β_3/β_5–doubly deficient mice; 2) despite the parallel roles of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in angiogenesis, $\alpha_v\beta_5$ and other integrins tested were not upregulated in response to β_3 deficiency in endothelial cells; and 3) enhanced VEGF-induced angiogenic responses in β_3-null mice may be due, at least in part, to the elevated VEGFR-2 levels observed in β_3-deficient endothelial cells.

Tumor angiogenesis is enhanced in β_3-deficient mice

Given that adult tumor angiogenesis can be inhibited by blockade of $\alpha_v\beta_3$ integrin[11–13,17], we investigated whether β_3 deficiency had any effect on adult tumor angiogenesis. β_3-null and wild-type mice were injected subcutaneously with murine tumor cells; either melanoma (B16F0) or lung carcinoma (CMT19T) cells. As shown in Fig. 1*a*, tumors not only grew in both lines, but tumor size was enhanced significantly in the β_3-deficient mice when compared with controls ($P < 0.02$ for B16F0 and $P < 0.01$ for CMT19T). Representative examples of B16F0 and CMT19T tumors are shown in Fig. 1*c*. The growth of a human melanoma (A375SM) cell line was also tested by subcutaneous injection into immunocompromised (Rag-2) mice and Rag-2/β_3-null mice (Fig. 1*b*). In concordance with the other tumor models, A375SM tumors were significantly larger in Rag-2/β_3-null mice ($P < 0.003$) (Fig. 1*b*).

Sections of tumors were stained with hematoxylin and eosin (H&E). Microvessels were evident in tumors in both wild-type and β_3-null mice (Fig. 1*d*). Immunostaining with antibodies to various endothelial markers, including vascular endothelial (VE)-cadherin, laminin-1 and platelet endothelial-cell adhesion molecule-1 (PECAM-1) (Fig. 1*e*), VEGFR-2 and Tek (data not shown), established that the blood vessels in the β_3-null mice expressed all of the tested markers of endothelial cells. Blood-vessel density was quantified by counting the number of vessels per unit area across entire tumor sections. β_3-null mice had significantly elevated numbers of vessels per square millimeter of tumor when compared with wild-type controls ($P < 0.03$) (Fig. 1*f*). Importantly, vessel density in non tumor-burdened adult scruff-skin was comparable between β_3-null and wild-type mice (Fig. 1*g*) suggesting that the enhanced tumor angiogenic response was not due to elevated blood-vessel density in untreated β_3-null skin.

β_3/β_5-deficient mice have enhanced tumor angiogenesis

Because $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are related receptors and are both thought to be involved in angiogenesis[15,20], it is possible

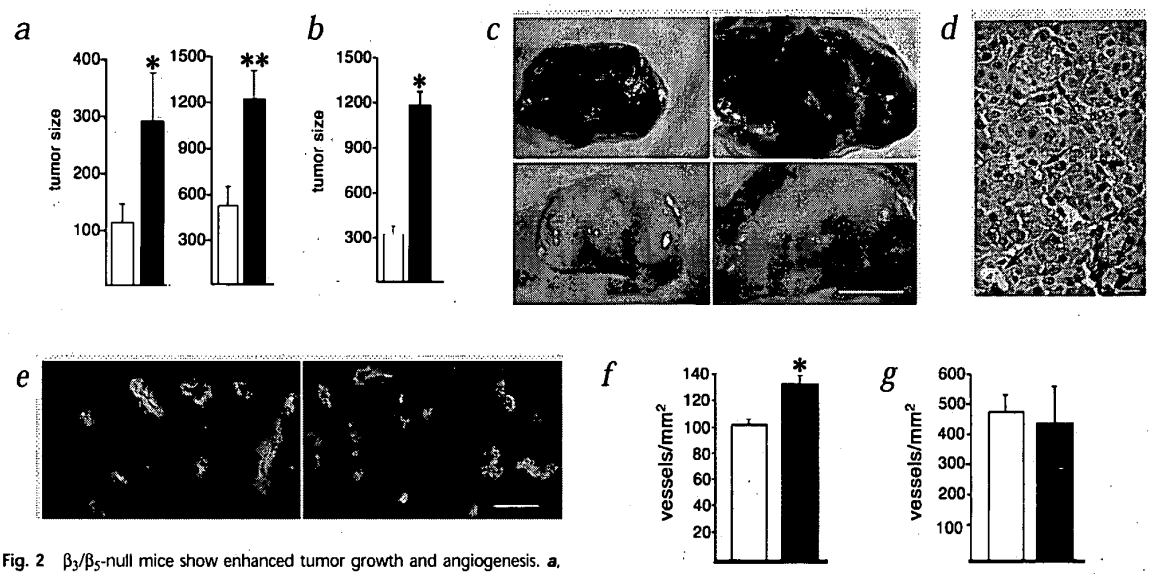


Fig. 2 β_3/β_5-null mice show enhanced tumor growth and angiogenesis. ***a***, Measurement of B16F0 (left) and CMT19T (right) s.c. tumors in wt (□) and β_3/β_5-null (■) mice. The mean tumor volume in mm^3 ± s.e.m. is given for 10-day-old tumors. *, $P < 0.05$; **, $P < 0.007$. ***b***, Measurement of human LS180 s.c. tumors grown in Rag-2 (□) and Rag-2/β_3/β_5-null (■) mice. The mean tumor weight in mg ± s.e.m. is presented for 21-day-old LS180 tumors (*, $P < 0.003$). ***c***, Macroscopic appearance of 10-day-old B16F0 (top) and CMT19T (bottom) tumors grown in control (left) and β_3/β_5-null (right) mice. ***d***, In H&E-stained sections of tumors from β_3/β_5-null mice microvessels are clearly visible (arrows). ***e***, Microvessels were detected by PECAM immunostaining of tumors in control (left) and β_3/β_5-null (right) mice. ***f***, Microvessel density in B16F0 tumors was significantly higher in β_3/β_5-null (■) mice than in wild-type (□) mice. *, $P < 0.008$. ***g***, In untreated skin sections vessel density was the same in wild-type (□) and β_3/β_5-null (■) mice. Scale bars: 1 cm (*c*); 50 μm (*d* and *e*).

that in the absence of both β_3 and β_5 integrins angiogenesis would be completely blocked. To test this, we examined subcutaneous B16F0 and CMT19T tumors in β_3/β_5–doubly deficient mice. Results showed that tumor growth was not only supported in these mice, but also that tumors were significantly larger when compared with controls (Fig. 2*a*). Similarly, subcutaneous growth of colon carcinoma (LS180) in Rag-2/β_3/β_5-null mice was significantly enhanced compared with controls ($P < 0.014$) (Fig. 2*b*). Representative examples of B16F0 and CMT19T tumors are shown in Fig. 2*c*. Histological analysis of an H&E-stained tumor section (Fig. 2*d*) and immunodetection of PECAM-1 (Fig. 2*e*), VE-cadherin, laminin-1 and VEGFR-2 (data not shown) revealed that angiogenic blood vessels were clearly detectable in the β_3/β_5-null mice. Moreover, tumors from these mice were significantly hyper-vascularized when compared with controls ($P < 0.008$) (Fig. 2*f*). In contrast, blood-vessel density in unchallenged β_3/β_5-null skin was comparable to control skin

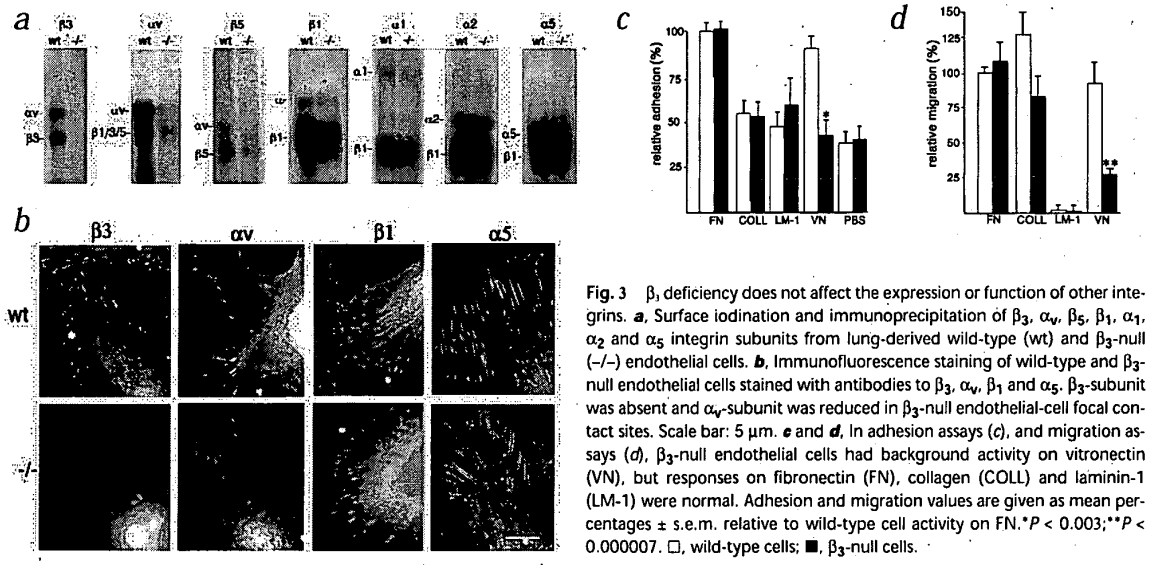


Fig. 3 β_3 deficiency does not affect the expression or function of other integrins. ***a***, Surface iodination and immunoprecipitation of β_3, α_v, β_5, β_1, α_1, α_2 and α_5 integrin subunits from lung-derived wild-type (wt) and β_3-null (–/–) endothelial cells. ***b***, Immunofluorescence staining of wild-type and β_3-null endothelial cells stained with antibodies to β_3, α_v, β_1 and α_5. β_3-subunit was absent and α_v-subunit was reduced in β_3-null endothelial-cell focal contact sites. Scale bar: 5 μm. ***c*** and ***d***, In adhesion assays (*c*), and migration assays (*d*), β_3-null endothelial cells had background activity on vitronectin (VN), but responses on fibronectin (FN), collagen (COLL) and laminin-1 (LM-1) were normal. Adhesion and migration values are given as mean percentages ± s.e.m. relative to wild-type cell activity on FN. *$P < 0.003$; **$P < 0.000007$. □, wild-type cells; ■, β_3-null cells.

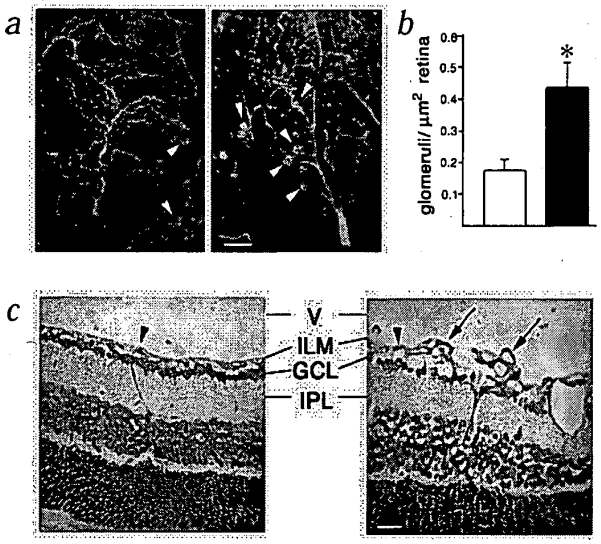


Fig. 4 Hypoxia-induced retinal angiogenesis is enhanced in β_3-null mice. ***a***, Flat mounts of retinas from wild-type (left) and β_3-null (right) mice that have undergone hypoxia-induced retinal angiogenesis show extensive areas of neovascularization with excessive development of vascular glomeruli (arrowheads) in the β_3-null retinas. ***b***, Quantification of vascular glomeruli in wild-type (□) and β_3-null (■) retinas (n = 9–12 mice per genotype; *, $P < 0.02$). ***c***, Cross-sections of eyes from wild-type (left) and β_3-null (right) mice showing retinal neovascularization internal to the inner limiting membrane in both genotypes (arrowheads) with excessive development of vascular glomeruli in the β_3-null retinas (arrows). V, vitreous; ILM, inner limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer. Scale bars: 100 µm (*a*); 50 µm (*c*).

(Fig. 2*g*). Thus, our data provide strong evidence that deficiency of either β_3 alone, or both β_3 and β_5 integrins, can enhance tumor growth and angiogenesis. These data demonstrate that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins are not essential for tumor angiogenesis.

β_3 deficiency does not affect other integrin subunits

Apart from the two receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, other integrins such as the fibronectin receptor $\alpha_5\beta_1$ and the collagen receptors $\alpha_2\beta_1$ and $\alpha_1\beta_1$ have also been suggested to play roles in angiogenesis[9–14,25]. This raises the possibility that other integrins may compensate for β_3 deficiency. We compared the expression profiles and functions of integrins in wild-type and β_3-null endothelial cells. Surface iodination followed by immunoprecipitation of β_3 and α_v integrins showed a complete loss of β_3 integrin and reduced levels of α_v integrin in the β_3-null endothelial cells. However, no changes in surface expression of β_5, β_1, α_1, α_2 or α_5 integrins were detected by surface iodination and immunoprecipitation (Fig. 3*a*). Western-blot analysis also showed no difference in total β_5-subunit levels in endothelial cells (data not shown). Focal contact distribution of integrins in the β_3-null endothelial cells was examined by immunofluorescence using antibodies to various integrins, including β_3, α_v, β_1 and α_5 integrins. Apart from the absence of detectable β_3 integrin in the β_3-null endothelial cells, α_v expression appeared to be somewhat reduced and there were no changes in β_1 or α_5 integrin patterns (Fig. 3*b*).

To test whether the loss of β_3 integrins had any effect on the functions of other integrins, we performed adhesion and migration assays on fibronectin, collagen, laminin-1 and vitronectin. In adhesion assays, wild-type and β_3-null endothelial cells adhered equally well to fibronectin, collagen and laminin-1. In contrast to wild-type cells, β_3-null cells showed background levels of adhesion to vitronectin (Fig. 3*c*). In migration assays, β_3-null endothelial cells had normal migratory abilities on most substrates; but migration was ablated on vitronectin (Fig. 3*d*). These data show no evidence for compensation by other integrins in response to β_3 deficiency.

β_3 deficiency enhances VEGF-induced blood-vessel growth

Since β_3-null mice exhibited enhanced tumor angiogenesis, we sought to investigate the effect of β_3 deficiency in another adult neovascularization assay. Hypoxia-induced retinal neovascularization in neonatal mice is a model for the ischemic disease retinopathy of prematurity, and it provides a useful

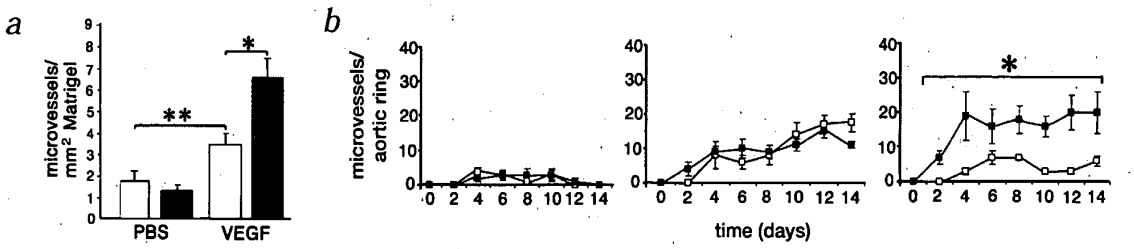
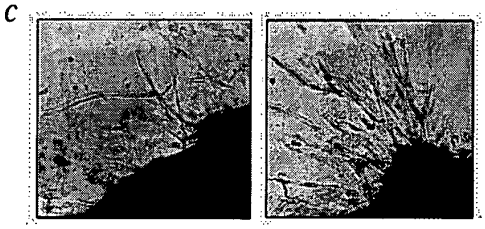


Fig. 5 β_3 deficiency enhances VEGF-induced vessel growth *in vivo* and *in vitro*. ***a***, Quantification of microvessels infiltrating Matrigel implants in wild-type (□) or β_3-null (■) mice in the presence or absence of VEGF shows augmented VEGF-induced angiogenesis in the β_3-null mice compared with VEGF-induced angiogenesis in wild-type mice. *, $P < 0.05$. VEGF-induced angiogenesis in wild-type mice was significantly enhanced compared with PBS controls. **, $P < 0.015$. ***b***, In *ex vivo* aortic ring assays microvessel numbers were counted from wild-type (□) and β_3-null (■) aortic rings grown in the presence of DMEM (left panel), FCS (middle) or VEGF (right). β_3-null samples had elevated angiogenic responses to VEGF (n = 5–7 mice per genotype; *, $P < 0.05$). ***c***, Low-power light micrographs of representative wild-type (left) and β_3-null (right) aortic ring microvessels grown in the presence of VEGF.



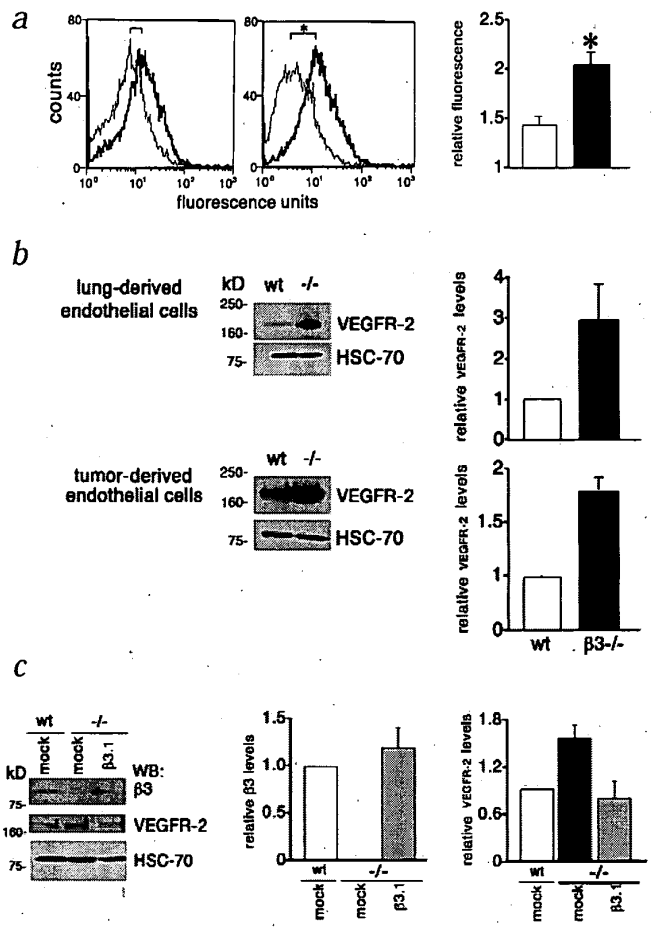


Fig. 6 Enhanced VEGFR-2 expression on β_3-null endothelial cells. ***a***, Flow-cytometric analysis of VEGFR-2 surface-expression levels showed that β_3-null (right) endothelial cells expressed significantly higher levels of VEGFR-2 relative to wild-type controls (left). Bold line, VEGFR-2-labeled cells; thin line, negative control. Bar chart shows means ± s.e.m. of relative surface VEGFR-2 expression in wild-type (□) and β_3-null (■) endothelial cells compared with negative control (n = 3; *, $P < 0.014$). ***b***, Western-blot analysis of VEGFR-2 levels in wild-type and β_3-null endothelial cells that were either grown in culture or isolated from tumors directly. Bar charts represent densitometry results (means ± s.e.m.) from 2–4 independent experiments that show that β_3-null (■) endothelial cells express 2–3-fold more VEGFR-2 than wild-type controls (□). Western blotting for HSC-70 provided loading controls. ***c***, β_3-null endothelial cells were infected with control retrovirus (mock), or with virus containing human β_3 integrin ($\beta_{3.1}$) and mock-infected wild-type cells were used as controls. Western-blot analyses confirmed that β_3 levels were restored in β_3-null cells infected with $\beta_{3.1}$. In β_3-null cells infected with $\beta_{3.1}$, VEGFR-2 expression was reduced to wild-type levels. Western blotting for HSC-70 provided loading controls. Bar charts represent densitometry results (means ± s.e.m., n = 3) of relative β_3 integrin and VEGFR-2 levels.

method to quantify neovascularization in β_3-null mice. Postnatal day 7 (P7) mice were placed in 75% oxygen for five days causing central avascularization of both wild-type and β_3-null retinas (data not shown). This incubation was followed by housing the mice for five further days (until P17) under normoxic conditions after which neovascularization was detected by perfusion of the entire vasculature with a non-diffusible fluorescein-dextran solution. In flat-mounted wild-type and β_3-null retinas, we detected areas of neovascularization and vascular glomeruli (Fig. 4*a*). Vascular glomeruli are highly proliferative clusters of tortuous vessels that are produced in response to angiogenic stimuli and protrude through the inner limiting membrane. The numbers of glomeruli were counted to compare retinal neovascularization in wild-type and β_3-null mice. We observed significantly elevated numbers of vascular glomeruli in β_3-null mice at P17 (Fig. 4*a* and *b*) and in retinal cross-sections (Fig. 4*c*). These data provide evidence that β_3-null mice show enhanced neovascularization not only in tumors but also in hypoxia-induced retinopathy.

VEGF expression is elevated in hypoxia-induced retinal angiogenesis and is thought to be the major angiogenesis-stimulating factor in this system[26,27]. Because hypoxia-induced retinal neovascularization was enhanced in the β_3-null mice, we hypothesized that VEGF-stimulated angiogenesis per se might be elevated in β_3-deficient mice. Matrigel implants impregnated with VEGF or PBS (in the presence of heparin[44]) were administered subcutaneously to wild-type and β_3-null mice and blood-vessel infiltration of the implants was quantified. In contrast to PBS controls, VEGF induced angiogenic responses in both wild-type and β_3-null mice and this response was significantly elevated in β_3-null mice when compared with controls (Fig. 5*a*).

Further analysis of VEGF-induced neovascularization responses was carried out using *ex vivo* aortic ring assays. Wild-type and β_3-null aortic rings were embedded in Matrigel in the presence or absence of serum or VEGF and the numbers of vascular sprouts per aortic ring over a period of 14 days in culture were assessed. In the absence of serum or VEGF, very little vessel outgrowth was detected in either wild-type or β_3-null samples, whereas serum induced neovascularization to a similar extent in both. However, in the presence of VEGF, the total number of vessel sprouts was significantly higher for β_3-null samples when compared with wild-type controls ($P < 0.05$) (Fig. 5*b*). Representative micrographs of vessels sprouting from aortic rings grown in the presence of VEGF are shown in Fig. 5*c*.

Elevated VEGFR-2 levels in β_3-null endothelial cells

Since β_3-null mice had elevated responses to VEGF, we tested whether the β_3-null endothelial cells had elevated levels of VEGFR-2. Flow cytometric analysis showed that β_3-null endothelial cells expressed significantly higher levels of surface VEGFR-2 than wild-type control endothelial cells ($P < 0.014$) (Fig. 6*a*) and densitometric analyses of western blots approximately three-fold more total VEGFR-2 (Fig. 6*b*). In addition, tumor-derived endothelial cells from β_3-null mice also had elevated levels of total VEGFR-2 (Fig. 6*b*). Tumor-derived endothelial cells from wild-type and β_3-null mice both had consistently elevated total VEGFR-2 levels when compared with lung-derived endothelial cells. Moreover, we showed that transduction of β_3-null endothelial cells with human β_3 integrin not only in-

duced β_3 expression in these cells, but also reduced the aberrant levels of VEGFR-2 to wild-type levels (Fig. 6*c*), suggesting that β_3 integrin can regulate VEGFR-2 expression.

Discussion

Our observations, that β_3-null or β_3/β_5-null mice not only support tumor angiogenesis and tumor growth, but even exhibit augmented responses, were unexpected and call for a reconsideration of the functions of α_v integrins in angiogenesis.

The integrin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, have been implicated in angiogenesis by their expression in vascular sprouts and by experiments blocking angiogenesis stimulated by growth factors, by tumors[11-13] or in hypoxia-induced retinal angiogenesis[16,19]. These data have been interpreted to suggest that antagonists of $\alpha_v\beta_3$ integrin inhibit angiogenesis by blocking its ligand-binding functions with consequences such as reduced proliferation and migration and increased apoptosis of endothelial cells[11-18]. Thus $\alpha_v\beta_3$ integrin has been thought to be essential for both developmental and pathological angiogenesis, and this has led to the generation of $\alpha_v\beta_3$ antagonists as potential anti-angiogenic therapeutics[21].

However, it is also known that mice lacking α_v integrins[22] and others deficient in β_3 or β_5 integrins[24,25], can support developmental angiogenesis. What was not known thus far is whether the absence of these integrins has any impact on pathological angiogenesis. In contrast with the inhibitor data, our results show not only that neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ integrin is required for such angiogenesis, but also that, in their absence, there is an increased angiogenic response. Furthermore, the enhanced angiogenic responses in the β_3-null and β_3/β_5-null mice lead to enlarged tumors, again in marked contrast with the blocking data where $\alpha_v\beta_3$ antagonists inhibit tumor growth. Our *in vivo* findings appear to be supported by recent *in vitro* observations that $\alpha_v\beta_3$ antagonists enhance the differentiation of endothelial cells into tubes[28]. In order to generate vessels, endothelial cells need to proliferate, survive and migrate into avascular tissue. As discussed above, these processes were previously thought to be dependent on $\alpha_v\beta_3$ integrin, but our data indicate that this is not the case. How can these discrepancies between the blocking and genetic ablation data be explained?

A first possibility is that genetic ablation underestimates the importance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins because of some sort of compensatory response by other adhesion molecules. We failed to detect upregulation in levels or activities of other integrins including $\alpha_v\beta_5$ in β_3-null endothelial cells. Additionally, β_3/β_5-null mice show the same enhanced tumor growth and angiogenesis as do β_3-null mice. This indicates that $\alpha_v\beta_5$ integrin is unlikely to compensate for the absence of $\alpha_v\beta_3$ integrin and that neither $\alpha_v\beta_3$ nor $\alpha_v\beta_5$ integrin are essential in angiogenesis. However, other mechanisms of compensation cannot be ruled out.

A second possible explanation for the discrepancy is that the blocking experiments may overestimate the contributions of $\alpha_v\beta_3$ in angiogenesis. Assessment of the impact of known $\alpha_v\beta_3$ antagonists such as LM609 (ref. 17) in β_3-null and wild-type mice would be of great interest. However, LM609 does not react with mouse $\alpha_v\beta_3$ and thus use of peptide antagonists may better address specificity of these reagents in future studies. Cross-talk between $\alpha_v\beta_3$ and other integrins such as $\alpha_5\beta_1$ has been suggested to affect angiogenesis[10,25]. Trans-dominant inhibition of other integrins by interference with β_3 integrins has also been demonstrated[29]. In fact, cross-talk among integrins is a well established phenomenon[30-33]. Based on these prior results, it is possible that the antibodies and low-molecular-weight antagonists of integrins, however specific they are, could be inhibiting other integrins, or even other functions, indirectly. Blocking and other experiments have highlighted the importance of α_5, α_1 and α_2 in angiogenesis[10,12-14,25,34] and it is clear that $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are not the only integrins involved in angiogenesis.

A third possible explanation could be an altered mechanism of angiogenesis in the β_3-null mice. Recent data have indicated that in pathological angiogenesis, some endothelial cells in newly formed blood vessels can originate from bone marrow–derived stem cells[35-38]. The differences between $\alpha_v\beta_3$ blocking and genetic ablation results may relate to differences in stem-cell mobilization. Notably, in a recent study of placental growth factor (PlGF)-deficient mice, developmental angiogenesis was normal, but loss of PlGF impaired pathological angiogenesis. Here the differences were attributed to inhibition of mobilization of bone marrow–derived endothelial cells in the PlGF-null mice[39]. While mobilization of bone marrow could be enhanced in β_3-null mice, such a possibility would not explain our *ex vivo* results and the precise mechanism would require further elucidation especially concerning the role of VEGFR-2. An alternative mechanism is that absence of α_v integrins could compromise recruitment of leukocytes and that this in turn could allow faster tumor growth.

Our observation that tumors are actually larger in mice lacking $\alpha_v\beta_3$ or both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and that angiogenesis is actually enhanced in these mice suggest that, rather than being required for angiogenesis, these integrins might normally have a role in limiting angiogenesis *in vivo*. To cite one potential example, during the growth of new vessels, ligated and unligated integrins might provide positive and negative signals for endothelial cell growth and/or survival, only allowing the formation of vessels in the presence of appropriate extracellular matrix ligands. Integrin antagonists could then mimic the unligated state and inhibit vessel growth. In this scenario, the absence of these integrins would result in a loss of the negative signals and could lead to enhanced angiogenesis, as we observed.

Our observation of elevated levels of VEGFR-2 expression on β_3-null endothelial cells suggests one mechanism by which $\alpha_v\beta_3$ integrin could negatively regulate angiogenesis. On the one hand, this could simply be some kind of angiogenic compensatory response to the absence of β_3 integrins; on the other hand, this result could reflect a mechanism normally in play. Indeed, these two possibilities could represent two aspects of the same mechanism. Interactions between β_3 integrins and VEGFR-2 have been reported[40,41]. If a normal function of $\alpha_v\beta_3$ (and perhaps also $\alpha_v\beta_5$) integrin were regulation of the VEGF/VEGFR-2 signaling pathway, then absence of $\alpha_v\beta_3$ could lead to its dysregulation. One possibility, among many that could be imagined, is that $\alpha_v\beta_3$ normally acts as a negative regulator of VEGFR-2 expression and function and ablation of $\alpha_v\beta_3$ causes upregulation of VEGFR-2 (as we observe). Inhibitors of $\alpha_v\beta_3$ could then act by causing dysregulation of VEGFR-2. Although beyond the scope of this study, these results raise some important questions. For example, considering the primacy of VEGF/VEGFR-2 signaling in angiogenic processes, the true function of $\alpha_v\beta_3$ could be to

act as a regulatory balance between inducing and inhibiting angiogenesis.

Here we present strong evidence that β_3 and β_3/β_5-null mice support and even show enhanced pathological angiogenic responses and tumor growth. Although blocking studies have implicated $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as having major roles in neovascularization, our findings demonstrate that these integrins are not essential for this process. The efficacy of $\alpha_v\beta_3$ antagonists in angiogenesis inhibition is not in dispute here—such inhibitors may yet prove to be effective therapeutics. However, our data indicate that a more thorough understanding of the precise mechanisms of action of these inhibitors and of the roles of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in angiogenesis is required.

Methods

Antibodies. Antibodies against VEGFR-2, PECAM, VE-cadherin, α_5 integrin and biotinylated antibodies against β_3 integrin were purchased from Pharmingen (Bedford, UK). Rabbit polyclonal antibodies against human β_3 and β_5 integrins were gifts from B. Coller and L. Reichardt, respectively. Rabbit polyclonal antibody against β_1 integrin was as described[42]. Antibody against α_v integrin was either purchased from Chemicon (Harrow, UK) or for immunofluorescence was a gift from E. Ruoslahti. LM609 was purchased from Chemicon. Antibody against laminin-1 was purchased from Sigma (Poole, UK). Antibody against the heat-shock protein HSC-70 was purchased from Autogen Bioclear (Wiltshire, UK). Conjugated secondary antibodies were purchased from Biosource (Nivelles, Belgium).

Extracellular matrix reagents. Fibronectin for cell culture was purchased from Sigma. Rat fibronectin for functional assays was purchased from Gibco BRL (Paisley, UK) and collagen, vitronectin, laminin-1 were obtained from Collaborative Biomedical Research (Bedford, Massachusetts).

Mouse transplants. Age- and sex-matched control and integrin-deficient mice on a mixed genetic background (C57BL6/129Sv) were given single s.c. injections of either 1×10^6 B16F0 or CMT19T cells and collected ten days after injection (n = 9–12 mice per group). Rag2- and Rag2/integrin-deficient mice were given single s.c. injections of either 2×10^6 LS180 or A375SM cells and collected 3–5 wk after injections (n = 3–11 mice per group).

Quantification of blood-vessel density. Blood-vessel density was quantified by counting the total numbers of PECAM$^+$/laminin-1$^+$ blood vessels across whole sections of tumors (n = 3–6 per group).

Primary lung endothelial-cell isolation. Wild-type and β_3-null mouse lungs were minced, collagenase-digested (Gibco), strained and the resulting cell suspension plated on flasks coated with a mixture of 0.1% gelatin (Sigma), 10 mg/ml fibronectin (Sigma) and 30 µg/ml Vitrogen (Collaborative Biomedical Research). Endothelial cells were purified by a single negative (FCγ-RII/III antibody; Pharmingen) and two positive (ICAM-2; Pharmingen) cell sorts using anti-rat IgG-conjugated magnetic beads (Dynal, Wiltshire, UK) producing a >97% pure population (J.C.L., unpublished data).

Immunofluorescence staining. Subconfluent cultures of endothelial cells and frozen tissue sections were immunostained as described[23].

Integrin-surface iodination and immunoprecipitation. These experiments were carried out as described[42,23].

Western blotting. Wild-type and β_3-null endothelial cells were lysed using RIPA buffer, and 100 µg of protein from each lysate were immunoprecipitated using the antibody VEGFR-2 followed by western-blot analysis also using an antibody against VEGFR-2 (ref. 40).

Cell migration and adhesion assays. Cell migration[43,23] and adhesion[23] assays were performed as described.

Hypoxia-induced retinal angiogenesis. This assay was carried out as described[16] using wild-type and β_3-null mice.

***In vivo* Matrigel plug assay.** This assay was carried out as described[44] in wild-type and β_3-null mice. Using 200 µl of growth factor-reduced Matrigel (Becton Dickinson, Beds, UK) containing 60 units/ml of heparin (Sigma), mixed with PBS or VEGF (60 ng/ml, R&D Systems, Oxon, UK). Blood-vessel infiltration in 7-day pellets was quantified by analysis of H&E-stained sections using a Zeiss Axioplan microscope.

Aortic ring assay. Mouse aortic ring assays were performed essentially as described[45]. 1-mm thoracic aortic rings were placed between 2 layers of 50 µl growth factor-reduced Matrigel supplemented with 20 U/ml heparin (Sigma), and overlaid with 100 µl of DMEM with or without VEGF (30 ng/ml). Microvessel outgrowth was visualized by phase microscopy and the number of vessels growing from each aortic ring was counted every 2 d using a Zeiss Axioplan microscope.

FACS analysis. Wild-type and β_3-null endothelial cells were incubated with VEGFR-2 antibody followed by incubation with a FITC-conjugated secondary antibody. Cells were then analyzed using a Becton Dickinson FACSCalibur flow cytometer.

Isolation of endothelial cells from transplants. CMT19T tumors grown subcutaneously in wild-type and β_3-null mice were collected 10 days after injection. Single-cell suspensions were generated by mincing the tumors and digesting them twice for 30 min at 37 °C with a mixture of collagenases (Roche, Lewis, UK; and Gibco). Cell suspensions were stained with an antibody against VE-cadherin (1 µg per 1×10^6 cells) followed by incubation with antibody against rat IgG-FITC. VE-cadherin$^+$ cells were sorted using a MoFlo (Cytomation, Frieburg, Germany) FACS sorter resulting in a ~90% pure population. 2×10^6 VE-cadherin positive endothelial cells were lysed in 200 µl RIPA buffer and used for VEGFR-2 immunoprecipitation as described above.

β_3 transduction. The medium from a cell line (E86β3.1) producing ecotropic retrovirus (gift from J. Marshall) was used to infect endothelial cells with functional human β_3 integrin. After 48 h endothelial cells infected with the β_3-integrin virus were selected from the non-infected cells by magnetic bead (Dynal) sorting using the antibody against human $\alpha_v\beta_3$, LM609. Cells were lysed and VEGFR-2 was immunoprecipitated followed by western-blot analysis as described. Experiments were repeated 3 times.

Animal regulations. All animals were used in accord with United Kingdom Coordination Committee on Cancer Research guidelines and Home Office regulations.

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